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Immunology

HLA-C is the inhibitory ligand that determines dominant resistance to lysis by NK1- and NK2-specific natural killer cells

(major histocompatibility complex/dimorphism/allorecognition/cytotoxicity)

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ABSTRACT **Natural killer (NK) cells recognize alloantigens on normal cells. One of these alloantigens correlates with homozygosity for a dimorphism of HLA-C at positions 77–80, which is shared by a number of *HLA-C* alleles. A second allelic alloantigen correlates with homozygosity for the alternative HLA-C dimorphism, which is shared by the remaining *HLA-C* alleles. Moreover, NK1- and NK2-specific NK cell lines can be generated by mixed leukocyte cultures in which donor and stimulator are homozygous for the alternative dimorphisms at positions 77–80 of HLA-C. In the present work, the role of HLA-C in NK cell-mediated allorecognition was directly investigated by analyzing the effects produced by transfection of several *HLA-C* alleles on NK sensitivity of class I-deleted mutant cell lines. Transfection of cells with *HLA-C* alleles encoding Asn-77–Lys-80 (including *HLA-Cw4*, *-Cw5*, and *-Cw6*) inhibited the lysis of the targets by NK1-specific NK cells, whereas *HLA-C* alleles encoding Ser-77–Asn-80 (including *HLA-Cw1*, *-Cw7*, and *-Cw13*) protected the targets from NK2-specific NK cells. Thus, *HLA-C* alleles are the dominant inhibitory ligands that protect targets from lysis by these allospecific NK cells.**

Natural killer (NK) cells lyse tumor and virally infected cells with no "restriction" by specific major histocompatibility complex (MHC) molecules on target cells (1, 2). On the contrary, NK cytotoxicity is generally inhibited by the expression of MHC class I molecules on the targets (3, 4). Thus, two types of recognition have been proposed for NK cells, which may be mediated by different receptor–ligand interactions: one leads to activation of NK cell lysis and the other leads to inhibition by MHC class I molecules (5).

Some NK cells have been recently shown to recognize allospecificities on normal allogeneic cells (6–9). Two of these allospecificities were mapped by family studies to the human MHC in close linkage with HLA-C (10–12). More precisely, NK1 specificity correlated with homozygosity for Ser-77–Asn-80 of HLA-C, whereas NK2 specificity correlated with homozygosity for the reciprocal dimorphism Asn-77–Lys-80 (12). Moreover, NK1-specific cells were reproducibly generated from individuals that were homozygous for Asn-77–Lys-80 by stimulation with target cells homozygous for Ser-77–Asn-80, whereas the reciprocal stimulation led to the generation of NK2-specific cells (13). Two hypotheses have been proposed to explain the correlation between NK-defined allospecificities and homozygosity for the HLA-C dimorphisms: (*i*) *HLA-C* is in close linkage with an unknown recessive gene that controls a target cell ligand that triggers NK cell killing. (*ii*) NK allorecognition is directly determined by *HLA-C*. In this case NK1-specific NK cells would be inhibited by *HLA-C* alleles encoding Asn-77–Lys-80, either homozygous or heterozygous (dominant resistance), thus recognizing only Ser-77–Asn-80 homozygous targets, whereas NK2-specific NK cells would be inhibited by *HLA-C* alleles encoding Ser-77–Asn-80, thus recognizing only Asn-77–Lys-80 homozygous targets.

The latter hypothesis is in agreement with the inhibitory role previously shown for class I molecules on NK cytotoxicity. In addition, a recent experiment has shown that transfection of *HLA-Cw3* may render a susceptible target resistant to clones with specificity 2 (14). However, *HLA-Cw3* can account for only a small number of the observed correlations between NK2 specificity and homozygosity for Asn-77–Lys-80. Thus, it cannot be excluded that NK1 specificities and some NK2 specificities might be due to the effect of other MHC genes in linkage with *HLA-C*. In addition, the protective effect of *HLA-Cw3* from lysis by human NK2-specific cells has been shown in a murine susceptible target, the mastocytoma cell line P815, but not in a human target, such as phytohemagglutinin (PHA)-activated blasts or B-cell lines.

To determine the role of HLA-C in NK-defined allospecificities, *HLA-C* alleles were transfected into the class I-deficient B-cell line 721.221, which is susceptible to both NK1- and NK2-specific NK cell lines. These new transfectants, together with *HLA-C* transfectants previously generated and the class I deletion mutant C1R that only expresses *HLA-Cw4*, were tested for susceptibility or resistance to NK1- and NK2-specific cell lines. The results presented clearly show that the *HLA-C* alleles examined are the ligands directly responsible for dominant inhibition of either NK1- or NK2-specific cells depending on the dimorphism at positions 77–80 of HLA-C.

MATERIALS AND METHODS

Cell Lines. AMALA, BM9, BM14, BOLETH, DBB, FPAF, HHKB, HO301, KASO11, LB, LG-2, PLH, and SPOO10 are HLA homozygous cell lines (HCL) from the Tenth International Histocompatibility Workshop (15). C1R is a HLA-A- and HLA-B-deficient Epstein–Barr virus (EBV)-transformed B-cell line, which expresses *HLA-Cw4* (16, 17). 721.221 (American Type Culture Collection) is a mutant EBV-transformed B-cell line expressing no HLA class I molecules (18). 721.221-B4-1, 721.221-B5-3 (provided by Terry Delovitch, Banting and Best Department of Medical Research, Toronto), E282, and E955 (provided by Dolores J. Schendel, Institute for Immunology, Munich) are 721.221 cell lines containing *HLA-Cw5*, *HLA-Cw13* (previously called *HLA-CwBL18*) (19), *HLA-Cw6*, and *HLA-Cw7* transgenes (20–23). K562 is a class I-null NK-susceptible tumor line.

Generation of Alloreactive NK Cell Lines. The generation of NK cell lines with NK1 and NK2 specificities has been

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Abbreviations: NK cells, natural killer cells; MHC, major histocompatibility complex; PHA, phytohemagglutinin; EBV, Epstein–Barr virus; E/T, effector/target cell.

described in detail (13). Briefly, highly purified $CD3^-$ $CD56^+$ cells [>95% by fluorescence-activated cell sorting (FACS) analysis] from *HLA-C* Asn-77–Lys-80 homozygous donors were cocultured with irradiated peripheral blood lymphocytes from *HLA-C* Ser-77–Asn-80 homozygous donors to generate NK1-specific cell lines. The reciprocal stimulation was performed to obtain NK2-specific cell lines. After 3 days of mixed lymphocyte culture, culture medium was supplemented with interleukin 2 (IL-2) (100 units/ml) and IL-2-containing supernatant (10%) and cultures were maintained for an additional 7 days. The resulting cell lines expressed CD56 but not CD3 by cytofluorometric analysis and lysed PHA blasts from the stimulating donor but not from the autologous donor by the ^{51}Cr release assay. Cells (10^5) were stained with fluorescein isothiocyanate-conjugated anti-CD3, phycoerythrin (PE)-conjugated anti-CD56 and anti-CD16 (Becton Dickinson) antibodies for 30 min on ice, washed, and analyzed in a FACScan (Becton Dickinson).

HLA-C Constructs and Transfections. To construct pRSV.5-Cw6 a 1.5-kb *Eco*RI fragment containing the *HLA-Cw6* cDNA was isolated from the plasmid pcEXV3-JTW1 (24), subcloned into Bluescript (Stratagene), reisolated as a *Sal* I/*Xba* I fragment and inserted into the pRSV-5 expression vector containing the Rous sarcoma virus promoter and the neomycin gene as a selectable marker (25). To construct pRSV.5-Cw1 a 3.6-kb *Xba* I fragment containing the *HLA-Cw1* gene was obtained from the plasmid pUC18-Cw1 (26) and ligated into the pRSV.5 vector. pRSV.5-Cw6 and pRSV.5-Cw1 were transfected by electroporation into 721.221 by using a gene pulser (Bio-Rad). After electroporation, cells were plated into six-well plates and selected after 2 days in G418-containing medium. Cell-surface expression of HLA-C was analyzed by FACS using the F4/326 monoclonal antibody (kindly provided by Soo Young Yang, Sloan–Kettering Memorial Hospital, New York) (24). Clones were derived from HLA-C-positive wells, expanded, and tested for the expression of HLA-Cw1 or -Cw6 by FACS.

Cytotoxicity Assays. The ^{51}Cr release assay was performed as described (13). Cell lines and transfectants were incubated for 60 min at 37°C with $Na^{51}Cr$ (100 μCi per 10^6 cells; 1 Ci = 37 GBq) (Amersham). Labeled cells were washed and 50-μl aliquots (5×10^3 cells per well) were seeded into 96 V-well plates. Effector cells were added at the indicated effector/target cell (E/T) ratios. After 4 hr at 37°C 100 μl of supernatant was collected from each well and the ^{51}Cr released into the supernatant was measured by γ-counting. Percentage specific cytotoxicity was calculated as described (13).

Unlabeled Target Inhibition Assay. ^{51}Cr-labeled target cells (5×10^3 cells) were added to wells containing unlabeled target cells at ratios ranging from 1:2.5 to 1:80, followed by addition of effector cells. E/T ratios were chosen so that percentage specific ^{51}Cr release (assayed as described above) was located in the linear portion of the cytotoxicity curve.

RESULTS

EBV-Transformed B-Cell Lines Are Specifically Recognized and Lysed By NK1- and NK2-Specific Cells. The capacity of NK cell lines to recognize allospecificities has been demonstrated by using PHA blasts as targets. However, PHA blasts are not suitable recipients for stable class I transfectants. Thus, the presence of NK-defined allospecificities was tested on EBV-transformed B-cell lines, which have been successfully used for class I gene transfections. $CD3^-$ $CD56^+$ cells (NK cells) from donors EB and ZM [*HLA-C* (Asn-77–Lys-80) homozygous] were primed with irradiated peripheral blood lymphocytes from donors TB and BS [*HLA-C* (Ser-77–Asn-80) homozygous], respectively. The resulting NK polyclonal populations (NK1-specific cell lines) were tested against a panel of *HLA* homozygous EBV-transformed B-cell lines by the ^{51}Cr release assay. High levels of cytotoxicity were found with homozygous B-cell lines expressing *HLA-Cw1* (LG-2), *-Cw3* (BOLETH, AMALA), *-Cw7* (BM14, HHKB), and *-Cw8* (HO301) [*HLA-C* (Ser-77–Asn-80) homozygous]. Low levels of lysis were detected with *HLA-Cw4* (BM9, FPAF), *-Cw5* (SPOO10), *-Cw6* (DBB, KASO11, PLH) positive cells [*HLA-C* (Ser-77–Asn-80) homozygous] (Fig. 1 *Left*). NK polyclonal populations derived from the reciprocal stimulations (NK2-specific cell lines) showed an opposite pattern of cytotoxicity, with a high level of lysis against target cells carrying *HLA-Cw4, -Cw5*, and *-Cw6* alleles [*HLA-C* (Asn-77–Lys-80) homozygous] and lower levels against *HLA-Cw1, -Cw3, -Cw7*, and *-Cw8* positive cells [*HLA-C* (Ser-77–Asn-80) homozygous] (Fig. 1 *Right*). These patterns of cytotoxicity substantially reproduced the ones displayed by NK1- and NK2-specific cell lines on PHA-activated T-cell blasts (13), indicating that the alloantigens recognized by alloreactive NK cells on PHA blasts are also expressed on B-cell lines and correlate with homozygosity for the reciprocal dimorphisms at positions 77–80 of HLA-C. Thus, since EBV-transformed B-cell lines behave as PHA blasts with respect to susceptibility/resistance to lysis by alloreactive NK cells, *HLA-C* transfectants in EBV B-cell lines may also be suitable targets for studying the mechanism of NK alloreactivity. However, NK-resistant EBV B-cell lines always showed some degree of lysis compared to NK-resistant PHA blasts, especially at

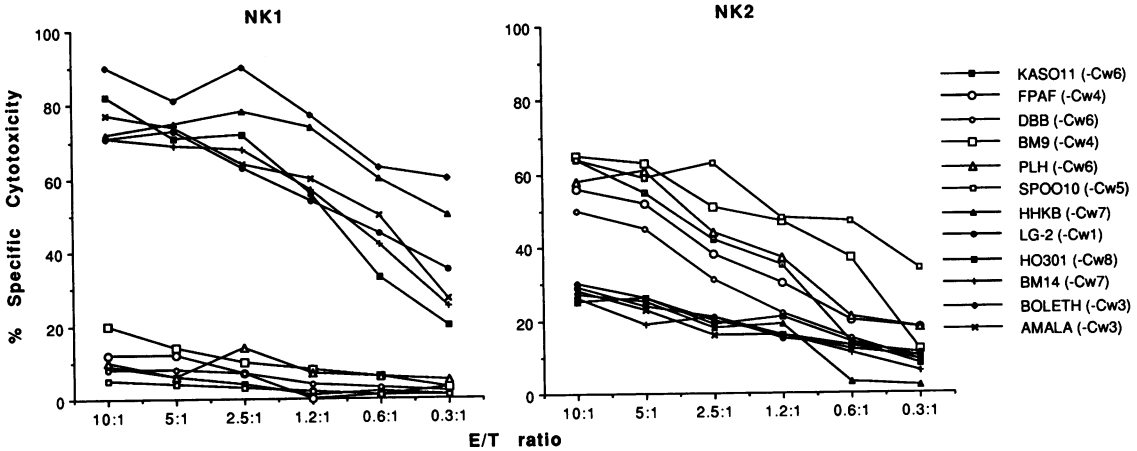

FIG. 1. Cytotoxicity of NK1- (*Left*) and NK2- (*Right*) specific cell lines against EBV-transformed *HLA* homozygous B-cell lines. NK cells were prepared as described (see text) and then assayed for cytotoxicity against ^{51}Cr-labeled target cells. Results are representative of those obtained in two separate experiments.

E/T ratios > 5:1. This nonspecific lysis might be related to a higher susceptibility of transformed cells to the non-MHC-restricted cytotoxicity of NK cell lines.

Cytotoxicity Profiles of NK1- and NK2-Specific Cell Lines on *HLA-C* Transfectants Reveal an Inhibitory Role of HLA-C on NK Cytotoxicity. To determine a direct role of HLA-C in NK cell-mediated alloreactivity, *HLA-Cw1* cDNA and *HLA-Cw6* genomic DNA were transfected into 721.221, an EBV-transformed B-cell line that does not express MHC class I molecules on the cell surface and is susceptible to lysis by NK cells (18). The parental cell line, the *HLA-Cw1* and *-Cw6* stable transfectants, the *HLA-Cw5, -Cw13, -Cw6, -Cw7* transfectants in 721.221 previously generated (20–23), and C1R, a mutant B-cell line that expresses only *HLA-Cw4* (16, 17), were analyzed for susceptibility to lysis by the NK1- and NK2-specific cells. Both displayed a high cytotoxic activity against 721.221 cells. Transfection of *HLA-Cw1, -Cw7,* and *-Cw13* alleles reduced the lysis of 721.221 by NK1-specific cells to only a small extent, whereas *HLA-Cw5* and *-Cw6* substantially affected the levels of cytotoxicity (Fig. 2 *Left*). In addition, C1R, which expresses *HLA-Cw4*, showed cytotoxicity levels similar to those of *HLA-Cw5* and *-Cw6* transfectants. Thus, among the *HLA-C* alleles tested, only *HLA-Cw4, -Cw5,* and *-Cw6* significantly reduced the lysis of deletion mutant cell lines by NK1-specific cells. Reciprocally, NK2-specific cells exhibited high cytotoxic activity against C1R (*HLA-Cw4*), *HLA-Cw5,* and *-Cw6* transfectants, while low levels of cytotoxic activity were detected against *HLA-Cw1, -Cw7,* and *-Cw13* transfectants, which, thus, protect from lysis by NK2-specific cells (Fig. 2 *Right*). These results demonstrate that *HLA-C* alleles sharing Asn-77–Lys-80 protected susceptible target cells from NK1-specific lysis, whereas alleles encoding the reciprocal dimorphism Ser-77–Asn-80 protected target cells from NK2-specific lysis. This allele-specific inhibition of NK1- and NK2-specific lysis was not related to differential expression of the transfected HLA-C molecules on the cell surface, as all transfectants showed similar levels of expression of HLA-C by flow cytometry.

HLA-C Molecules Do Not Inhibit Alloreactive NK Cells in Trans. To determine whether or not inhibitory *HLA-C* alleles expressed on bystander cells can also protect susceptible target cells from alloreactive NK lysis—i.e., protection in trans—target cells susceptible to NK lysis were labeled, mixed with either resistant or susceptible unlabeled cells, such as *HLA-C* transfectants and *HLA* homozygous cell lines, and tested for susceptibility to lysis by effector cells (cold target inhibition assay). Lysis of labeled cells from a Ser-77–Asn-80 homozygous donor (MW) by a NK1-specific polyclonal population [responder: donor RR, *HLA-C* (Asn-77–Lys-80) homozygous; stimulator: donor MW, *HLA-C* (Ser-77–Asn-80) homozygous] was unaffected in the presence of NK1-resistant unlabeled targets [transfectants and homozygous cell lines with *HLA-C* (Asn-77–Lys-80) alleles] (Fig. 3 *Left*). On the contrary, the addition of targets susceptible to NK1-specific cells, such as untransfected 721.221 cells, transfectants, and *HLA* homozygous cell lines with *HLA-C* (Ser-77–Asn-80) alleles resulted in a dose-dependent inhibition of lysis due to a competition between labeled and unlabeled target for lysis by NK1-specific cells. Similarly, lysis of labeled cells from an Asn-77–Lys-80 homozygous donor (RR) by a NK2-specific polyclonal population [responder: donor MW, *HLA-C* (Ser-77–Asn-80) homozygous; stimulator: donor RR, *HLA-C* (Asn-77–Lys-80) homozygous] was not inhibited by the addition of resistant unlabeled targets expressing the inhibitory ligands [transfectants and homozygous cell lines with *HLA-C* (Ser-77–Asn-80) alleles], whereas 721.221 cells, *HLA-C* transfectants, and *HLA* homozygous cell lines with *HLA-C* (Asn-77–Lys-80) alleles effectively competed for lysis by NK2-specific cells (Fig. 3 *Right*). Thus, cells expressing *HLA-C* alleles protecting against NK lysis did not prevent the lysis of the susceptible target in trans, even at very high labeled/unlabeled target ratios (1:80).

Cold target inhibition was also carried out by mixing labeled susceptible targets with another unlabeled nonspecific target, the erythroleukemia cell line K562. Again the unlabeled target competed with specific targets for lysis by NK1- and NK2-specific cell lines in a dose-dependent fashion (Fig. 4).

DISCUSSION

The present data directly demonstrate that resistance or susceptibility to lysis by alloreactive NK cells is controlled by *HLA-C* and not by a closely linked gene. In addition, comparison of the effects of several *HLA-C* alleles on NK sensitivity of class I-deleted mutant cell lines indicates that the dimorphism at positions 77 and 80 is critical to this NK-regulatory function of *HLA-C*. Transfection of a class I-deleted B-cell line (721.221) with *HLA-Cw5* and *-Cw6* alleles, which share Asn-77–Lys-80, rendered the target resistant to lysis by NK1-specific cells, without affecting susceptibility to lysis by NK2-specific cells. Transfection of the same target cell with *HLA-Cw1, -Cw7,* and *-Cw13* alleles, which share Ser-77–Asn-80, had the opposite effect (increased resistance to NK2-specific cells and susceptibility to NK1-specific cells). Another class I-deleted mutant cell line (C1R), which expresses only *HLA-Cw4* (Asn-77–Lys-80), was resistant to NK1- and susceptible to NK2-specific lysis. It was previously demonstrated that transfection of *HLA-Cw3* (Ser-77–Asn-80) into the murine mastocytoma cell line

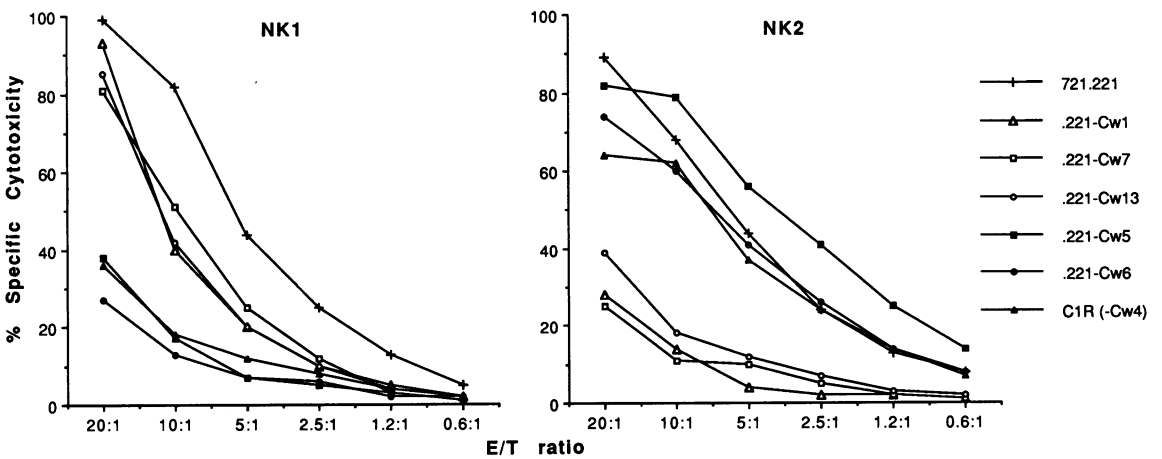

FIG. 2. Lysis of 721.221 cell lines containing *HLA-C* transgenes and of the cell line C1R by NK1- (*Left*) and NK2- (*Right*) specific cell lines. Results are representative of those obtained in three separate experiments.

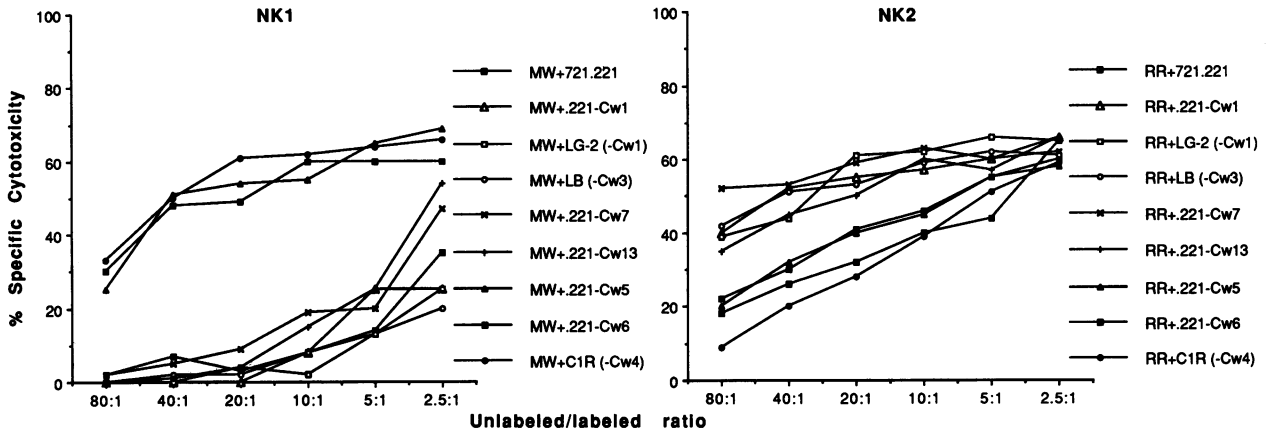

FIG. 3. Effect of addition of specific unlabeled target cells on NK1 (*Left*) and NK2 (*Right*) cytotoxic activity against ^{51}Cr-labeled targets. E/T ratio was 5:1 and 10:1 for experiments shown in *Left* and *Right*, respectively. Results are representative of three separate experiments.

P815 induces resistance to specificity 2 (equivalent to NK2) but not to specificity 1 (equivalent to NK1) (14). Thus, all the transfected *HLA-C* alleles display an inhibitory activity on the cytotoxicity of either NK1- or NK2-specific cells, according to the dimorphism at positions 77–80. This conclusion was confirmed by cold target inhibition experiments. Lysis of a labeled NK1 target (Ser-77–Asn-80 homozygous) was competitively inhibited by an excess of unlabeled transfectants and by *HLA* homozygous B-cell lines expressing *HLA-C* alleles with Ser-77–Asn-80 (including *HLA-Cw1*, *-Cw3*, *-Cw7*, and *-Cw13*). However, unlabeled cells expressing *HLA-C* alleles of the Asn-77–Lys-80 group did not function as competitive inhibitors, indicating that they were resistant to lysis by NK1-specific cells. Similarly, lysis of a labeled NK2 target (Asn-77–Lys-80 homozygous) was competitively inhibited by an excess of unlabeled cells expressing *HLA-C* alleles with Asn-77–Lys-80 (including *HLA-Cw4*, *-Cw5*, and *-Cw6*). Unlabeled cells with *HLA-C* alleles of the Ser-77–Asn-80 group were not able to competitively inhibit, indicating that they were resistant to lysis by NK2-specific cells. It is noteworthy that, in cold target inhibition experiments, susceptible targets are lysed even in the presence of a large excess of unlabeled targets expressing inhibitory *HLA-C* alleles. Thus, these alleles protect the resistant cells on which they are expressed but do not turn off completely the NK cell, which is then able to move to other cells and lyse susceptible targets. Finally, lysis of NK1- and NK2-susceptible targets by both alloreactive NK cell lines was inhibited by an excess of unlabeled tumor cell lines, such as K562, indicating that classical NK targets are also susceptible to lysis by alloreactive NK cells.

The inhibitory function of HLA-C on the cytotoxicity of alloreactive NK cells is in agreement with previous evidence that expression of MHC class I molecules protects from NK lysis (3, 4). The mechanisms by which HLA-C protects target, cells from NK recognition are still undefined. According to the "effector inhibition" model, HLA-C antigens might interact with NK receptors, delivering a negative regulatory signal to the NK cell. This model is supported by recent studies on the Ly49 cell-surface antigen in mouse. Ly49 is a membrane protein with an extracellular C-type lectin domain expressed by a subpopulation of murine NK cells, which has been shown to function as an inhibitory receptor specific for the MHC class I molecule H-2D^d (27). In human, two NK antigens have been proposed as inhibitory receptors for HLA-C, because they are expressed on alloreactive NK clones and correlate with NK-defined allospecificities (28).

In the "masking" hypothesis HLA-C might associate with target cell structures capable of triggering the NK cells; as a result, these structures would be modified or made inaccessible for NK recognition. This model is supported by previous detection of class I-associated molecules, including peptide hormone receptors (insulin and epidermal growth factor receptors) (29–31), CD25 (32), and CD8 (33). These or other molecules may function as NK target structures.

The role of amino acid residues 77 and 80 in the NK inhibitory function of HLA-C may be interpreted in two ways. Crystallographic models proposed for HLA-A2,

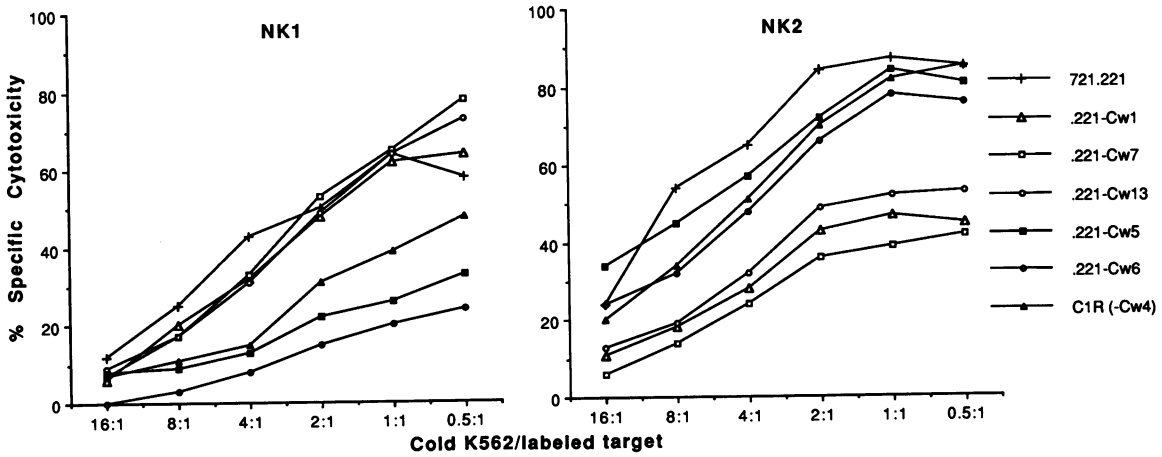

FIG. 4. Effect of addition of unlabeled K562 on NK1 (*Left*) and NK2 (*Right*) cytotoxic activity against ^{51}Cr-labeled targets. E/T ratio was 5:1 and 10:1 for experiments shown in *Left* and *Right*, respectively. Results are representative of those obtained in two separate experiments.

-Aw68, and -B27 suggest that these residues are located in the peptide binding cleft of HLA-C and, more specifically, in or near the F pocket, which binds the C-terminal amino acid residue of a peptide nonamer (34). Thus, HLA-C may bind peptides capable of mediating NK inhibition. A similar hypothesis has been previously proposed to explain the observations that amino acid variations in the peptide binding cleft of HLA-A2 play a role in converting susceptible target cells to NK-resistant cells (35) and that incubation with exogenous peptides increases the lysis of the targets to which they bind (36, 37). As the F pocket of HLA-C is characterized by a dimorphism (Asn-77–Lys-80 or Ser-77–Asn-80) two types of HLA-C-bound peptides characterized by different residues at position 9 might be bound to HLA-C, one inhibiting NK1-specific cell lines and the other inhibiting NK2-specific cell lines. However, the motifs of HLA-C-bound peptides, recently determined by pooled peptide sequencing, showed no correlation between amino acids at residue 9 and the dimorphism at positions 77–80 of HLA-C (23). A detailed analysis of single peptide sequences may be necessary to identify NK inhibitory peptides, if they occur.

MHC class I molecules are present on the cell surface in two forms: one is the β_2-microglobulin-associated form and the other is a free heavy chain (which may occur as a dimer) (38–44). The equilibrium between these conformations on the cell surface can be modified by incubating cells with peptides or β_2-microglobulin, which stabilize the β_2-microglobulin-associated form. Recent studies suggested that class I molecules may mediate protection from NK recognition in the free heavy-chain form, whereas the β_2-microglobulin-associated form leads to increased NK susceptibility (45). If this model were correct, it could be extended to the NK inhibitory function of HLA-C. HLA-C is characterized by a low affinity for β_2-microglobulin and, thus, may be significantly expressed on the cell surface in the free heavy-chain form (26, 46–50). In this model, residues 77 and 80 of HLA-C heavy chain could directly engage a NK inhibitory receptor or associate with a hypothetical NK target, thereby masking it. In either case, the NK receptor or the target structure interacting with HLA-C should display a limited polymorphism correlating with the HLA-C dimorphism at positions 77 and 80.

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